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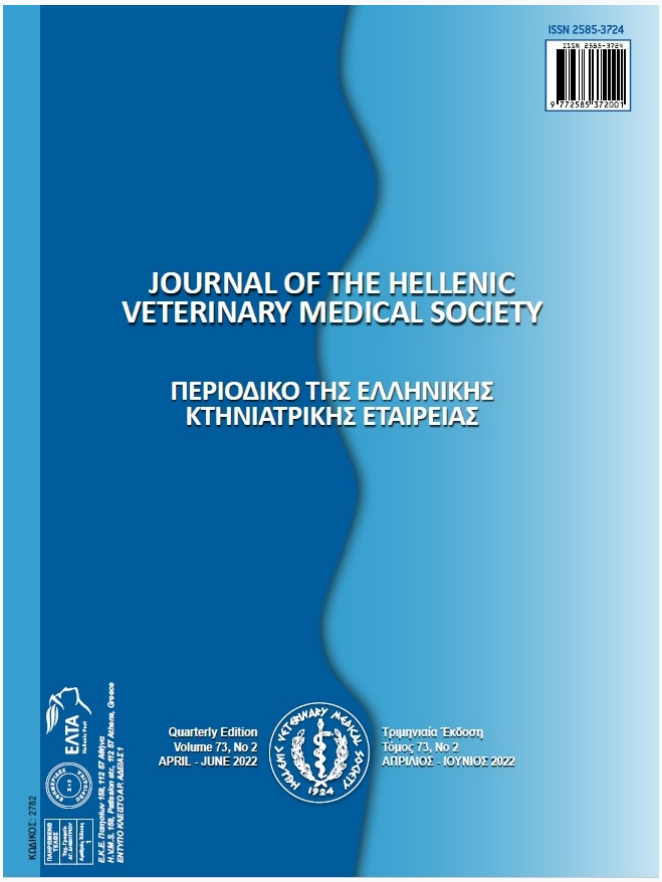

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Research article
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Determination genetic variation and phylogenetic analysis in *Echinococcus granulosus* isolated from Iraqi sheep

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ABSTRACT : *Echinococcus granulosus* causes cystic echinococcosis in intermediate hosts, such as sheep, cattle, goats, camels, and horses. Cystic echinococcosis is an essential parasitic disease that affects humans and animals and constitutes a significant public health and economic problem worldwide. This study aimed to determine the molecular characterization and phylogenetic analysis of *Echinococcus granulosus* in sheep's livers and lungs. The genetic variation was performed by directly sequencing the mitochondrial DNA (mtDNA) genes coding of the NADH dehydrogenase subunit 1 (ND1). The evidence from molecular studies and DNA sequencing revealed that the isolates of *Echinococcus granulosus* in Kirkuk city are related to the sheep strain G1 genotype. The alignment analysis showed that there is a concordance between isolates with each other by 99%. When comparing the results with Genbank, it indicated the occurrence of genetic variations between nucleotides represented by eleven codons, seven of which represented transversion mutations, and four were transition mutations.

Keywords: *Echinococcus granulosus*, *Cystic Echinococcosis*, Sheep,ND1 gene, Sequencing

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INTRODUCTION

Echinococcus granulosus (*E. granulosus*) is one of the smallest parasitic tapeworms that dwell as adult worms in the small intestine of the definitive hosts (dogs, wolves, hyenas, leopards, and lions), and as hydatid cysts in the intermediate hosts (sheep, goats, cattle, camels, buffalos, horses, donkeys, pigs, rabbits and humans) (Abdulla *et al.,* 2020; Hammad *et al.,* 2018; Hasan *et al.,* 2016). *E.granulosus* causes a zoonotic parasitic disease called cystic echinococcosis (CE). CE also called echinococcosis, hydatidosis and unilocular hydatidosis. CE is a severe epidemic disease that affects humans and causes significant economic losses to livestock. This disease spreads almost all over the world. Still, it is more common in rural areas, especially in grazing areas. The reason is due to the presence of the intermediate host (cattle and sheep) and the definitive host (dogs), which helps to complete the life cycle of this parasite (Alvi *et al.,* 2020; Kadhimand & Al-Mayali 2020; Mahdi *et al.,* 2020). CE grows and develops slowly, as the disease is characterized by the absence of symptoms in the early stages of infection. Symptoms depend on the affected organ, the liver being the most affected organ, with a rate of about 60-70%, followed by the lungs 20-22%, spleen, heart, muscles, eyes, thyroid, kidneys, brain, and bones (Saadi 2020; Sharma *et al.,* 2013). Some studies have recorded more than 50 cases per 100.000 people per year in endemic areas. The disease also kills about one million people annually worldwide and causes a loss of about three billion US$ (Fallahizadeh *et al.,* 2019; Alsaady and Al-Quzweeni 2019). Based on nucleotide sequences analysis of two mitochondrial regions, cytochrome c oxidase subunit 1 (CO1) and NADH dehydrogenase subunit 1 (ND1) and one ribosomal region, internal transcribed spencer 1 (ITS1), ten distinct genotypes or strain (G1-G10) have been described for *E.granulosus*. This includes sheep strain G1, Tasmanian sheep strain G2, buffalo strain G3, horse strain G4, cattle strain G5, camel strain G6, pig strain G7, cervid strain G8, the human polish strain G9 and the fennoscandian cervid strain G10 (Lavikainen *et al.,* 2003; Piccoli *et al.,* 2013; Yan *et al.,* 2018). Iraq is an endemic country for CE due to the spread of stray dogs infected with parasites. Although there are methods available to control the disease, it still remains a significant health problem. This investigation aimed to characterize the genetic variation and phylogenetic analysis of *E.granulosus* in terms of ND1 gene isolated from sheep liver.

MATERIALS AND METHODS

Sample collection

Eight samples of hydatid cysts isolated from sheep livers and lungs were used in this study. The samples were obtained from the Kirkuk slaughterhouse from 10/29/2019 to 2/16/2020 under the supervision of veterinarians (Figure 2). The ages of the animals ranged from 7 months to 1 year. The samples were transported by icebox to preserve the vitality of the protoscoleces to the laboratory (Figure 1).

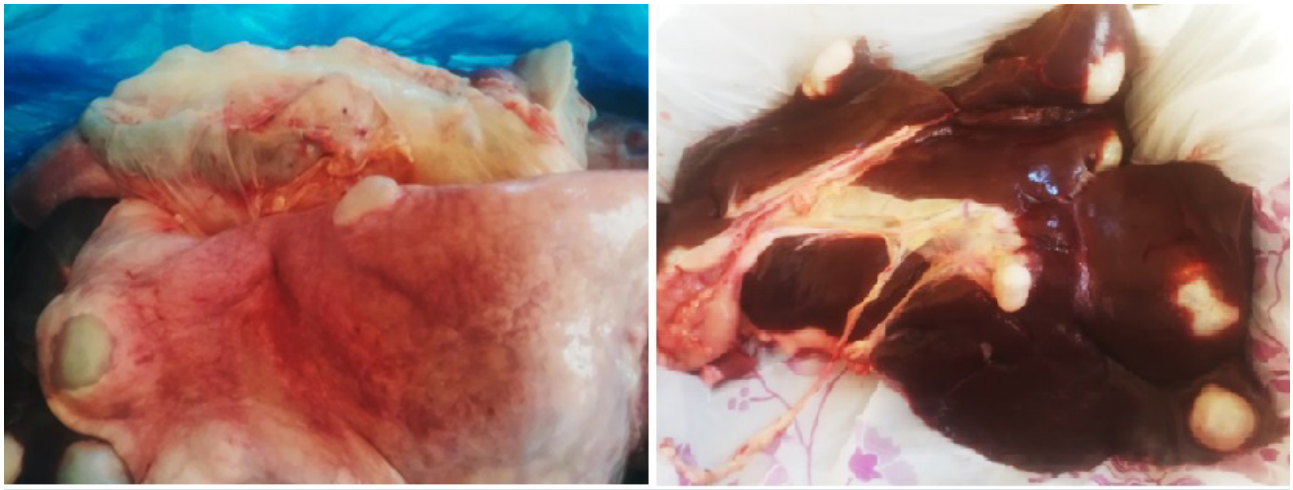

Figure 1: Hydatid cysts obtained from sheep's livers and lungs

Figure 2: The geographical area from which the samples were collected

Isolation of protoscoleces

Protoscoleces were isolated according to the Smyth (1985) method. Hydatid cysts were cleaned with 70% ethyl alcohol and washed with phosphate buffer saline (PBS), and then hydatid fluid was withdrawn from the cyst. Germinal Layer was extracted and washed with PBS. The hydatid fluid was withdrawn and centrifuged at 3000 rpm for 15 minutes. The filtrate was removed, the precipitate was taken, and then washed with PBS. The samples were stored at -20 °C.

DNA extraction from protoscoleces

DNA extraction was done for each sample using Genomic DNA Mini Kit (Wizard, Korea) according to the manufacturer's instructions. The purity and concentration of extracted DNA samples were analyzed by Nanodrop.

PCR assay

The PCR was performed in a total of 25µl, which contained 12.5µl Go Tag Green Master Mix (Promega, USA), 1µl of 10 pmol/µl of each primer, 2µl of the genomic DNA, and 8.5µl nuclease-free water. PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 30 sec and elongation at 72 °C for 30 sec and a final extension at 72 °C for 5 min. To amplified the ND1 gene (800 bp) was used forward (5′-GTTTTTGGGTTAGTCTCTGG-3′) and reverse (5′-ATCATAACGAACACGTGG-3′) primers (Sánchez *et al.,* 2012). PCR products were separated at 2% agarose gel in 80 V for 60 min. The gel was stained by ethidium bromide and results were checked under ultraviolet light.

Sequences and phylogenetic analysis

The PCR products of the ND1 gene for eight samples were sequenced. The DNA sequencing was performed by Macrogen Corporation (Korea) using the 3730 XL DNA Analyzers (Applied Biosystems, Foster City, California, USA). The reference sequences of the ND1 gene of *E.granulosus* were obtained from GenBank (https://www.ncbi.nlm.nih.gov/). Reference and isolate sequences were used in multiple alignments, and phylogenetic distances were calculated using BioEdit software. Phylogenetic trees were constructed using the neighbor-joining methods in MEGA version 6.0. All sequences were uploaded to GenBank and assigned GenBank number MW980133, MW980134, MW980135, MW980136, MW980137, MW980138, MW980139 and MW980140.

RESULTS

800 bp of PCR product was amplified of the ND1 gene (Figure 3). All eight samples were genotype G1 strain (100%). Alignment analysis demonstrates the similarity of 99% (for 6 samples) and 100% (for 2 samples). When comparing the results with Genbank, it shows the occurrence of mutations between nucleotides represented by eleven codons, 7 of which are transversion mutations and 4 transition mutations. In sample A, four mutations were found, three of which were transversion mutations and one transition mutation. Mutations were; (T\G→TGT\GGT) at site [572], (T\G→TTG\TGG) at site [574], (G\A→AGA\AAA) at site [617] and (T/G→AGT\AGG) at site [651]. The predicted effect was missense with anidentical ratio of 99% (Table 1). No mutation was found in sample B; the identical percentage was 100%. Sample C showed three transversion mutations in the site [607] (G\C→GAG\CAG), (G\T→AGT\ATT) in site [650] and (T\A→TTT\ATT) in site [673]. The mutation was an effective missense, and the identical rate between them was 99%. The percentage of congruence was 100% in sample D and no mutation was found. In sample E, one transversion mutation G\T→CAG\CAT) was identified in the site [150]. The mutation was missense, with anidentical percentage of 99%. About samples (F, G, H), transition mutation (T\C→TAT\

TAC) was detected at sites [285]. The mutation was silent and the identical was 99%. The phylogenetic tree results showed identical between samples (G, H, F) as 100%, also, the identical between samples (D, B) were 100%. The identical was between samples (A, C, E) as %99, whereas the identical between samples (D, B) and samples (H, F, G) were 99% (Figure 4, Figure 6).Based on a comparison between the sequences of ND1 gene for Iraqi *E.granulosus* isolates with other strains from different countries were 99% match with the common sheep strain (G1). It appeared that the highest percentage of identical (99%) was with China, Nigeria, Algeria, Greece, Tunisia, India, Mexico, Kazakhstan, Iran, Mongolia, Argentina, Brazil, Chile, Turkey, and Pakistan. While the countries samples were identical to each other by 100% as well (Figure 5).

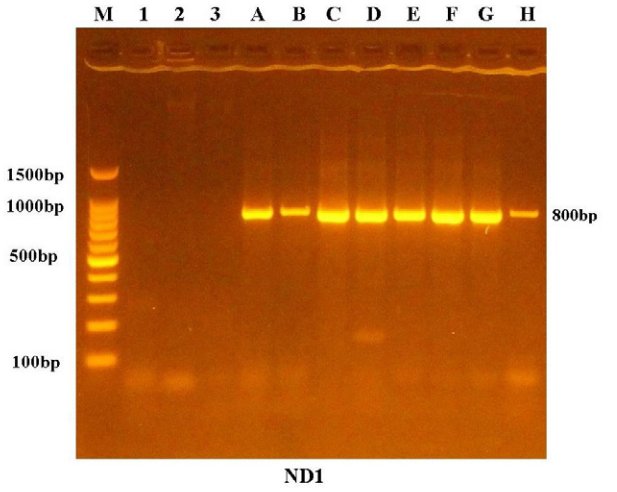

Figure 3: PCR product of ND1 gene

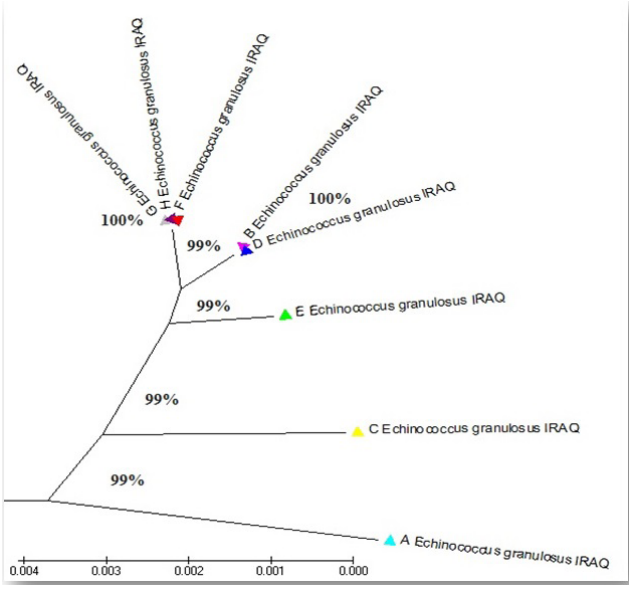

Figure 4: Neighbor-joining tree showing the identical ratio between the species of isolates

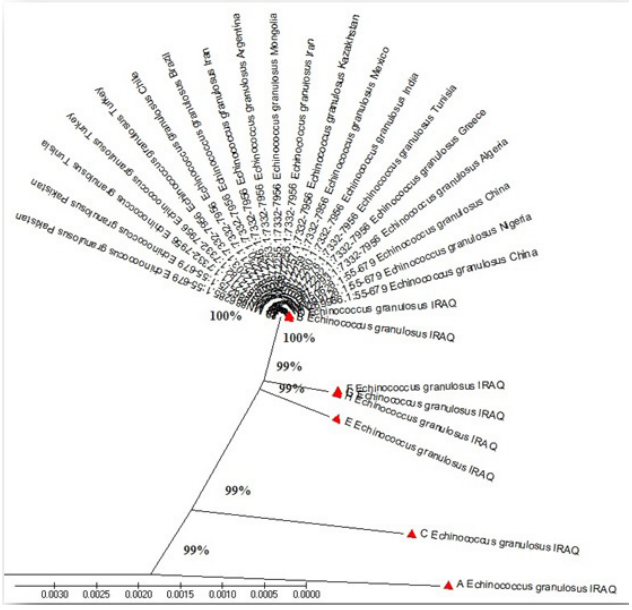

Figure 5: Neighbor-joining tree showing congruence between Iraqi isolates compared to global isolates of E.granulosus

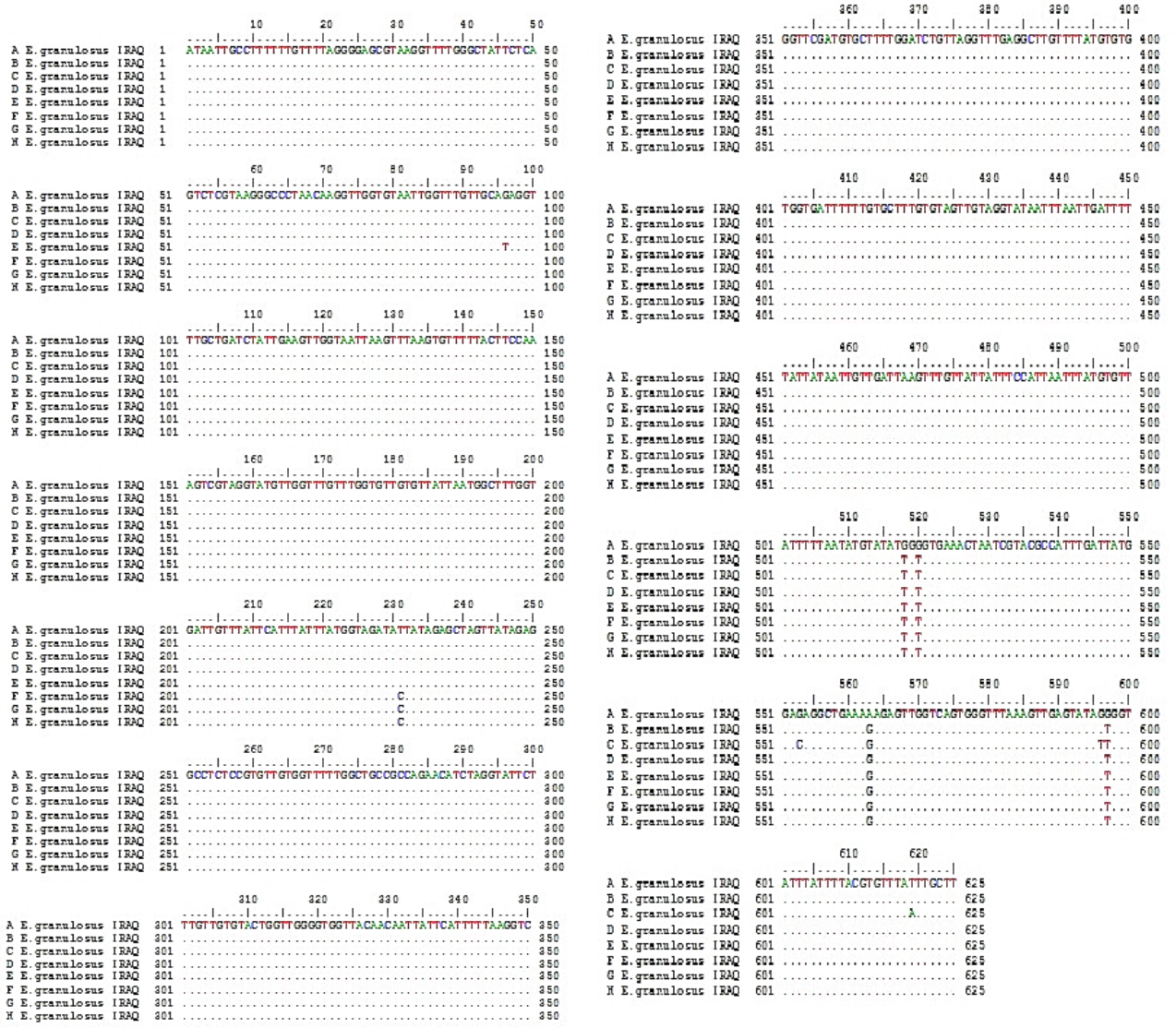

Figure 6: Multiple sequences alignment of ND1 gene

Table 1: Genetic variations of E.granulosus according to ND1 gene

Sample Number	Type of substitution	Location	Nucleotie	Nucleotide change	Predicted effect	Sequence ID with compare	Identities
A	Transversion	572	T\G	TGT\GGT	Missense	ID: MN269986.1	99%
	Transversion	574	T\G	TTG\TGG	Missense		
	Transition	617	G\A	AGA\AAA	Missense		
	Transversion	651	T\G	AGT\AGG	Missense		
B	----------	----------	----------	---------	----------	ID: MN269986.1	100%
C	Transversion	607	G\C	GAG\CAG	Missense	ID: MN269986.1	99%
	Transversion	650	G\T	AGT\ATT	Missense		
	Transversion	673	T\A	TTT\ATT	Missense		
D	----------	----------	----------	---------	----------	ID: MN269986.1	100%
E	Transversion	150	G\T	CAG\CAT	Missense	ID: MN269986.1	99%
F	Transition	285	T\C	TAT\TAC	Silent	ID: MN269986.1	99%
G	Transition	285	T\C	TAT\TAC	Silent	ID: MN269986.1	99%
H	Transition	285	T\C	TAT\TAC	Silent	ID: MN269986.1	99%

DISCUSSION

Although cystic hydatidosis gives rise to significant and critical public health and economic problems in Africa, Asia, and the Middle East, current efforts to control the disease are inadequate (Wen *et al.,* 2019). Because of the wide variety of cystic larval stages. Moreover, the control of hydatidosis is usually hampered due to ignorance of the true prevalence of the disease and the invalidity they cause (Saadi 2020). In addition, accurate diagnosis of hydatid cyst disease is often difficult because some of the esoteric diseases resemble those found in patients with hydatidosis (Kern *et al.,* 2017). Therefore, there has been a great need for a more reliable laboratory test to diagnose hydatidosis in this area. The PCR amplification and DNA sequencing of specific regions of the ND1 gene have been shown to provide reliable alternatives to more traditional methods for the specific identification of *Echinococcus* strains from different intermediate hosts (Nakao *et al.,* 2013). Based on the results above, it can be suggested that G1 or sheep genotype is the most important variant of *E.granulosus*. This finding should be considered during the planning and implementation of the *E.granulosus* control program region. The results demonstrated in this investigation agree with Iraqi isolates. Hamoo and Abdulraheem (2019) showed that the local isolate is G1 genotype (sheep strain), and the phylogenetic results were compatible with North Africa and the Middle East isolates as 95% and 96%.Fadhil & A'aiz (2016) reported three strains of the *E.granulosus* parasite, sheep strain (G1) 40%, buffalo strain (G3) 48% and camel strain (G6) 12%. Whereas, sheep were G1 (80%) and G3 (20%). Mustafa *et al.,* (2019), AL-Asadi *et al.,* (2021), Muhaidi *et al.,* (2017), Muhaidi *et al.,* (2018) identified *E.granulosus* in terms of ND1 gene as G1 genotype sheep strain (100%). The Iraqi isolates are consistent with the isolates of other countries, such as, Turkey (Barazesh *et al.,* 2019), Iran (Barazesh *et al.* 2019; Hajialilo *et al.,* 2012; Pezeshki *et al.,* 2013; Sharbatkhori *et al.,* 2016), Sudan (Ahmed *et al.,* 2018), Egypt (Amer *et al.,* 2015), Uzbekistan (Kim *et al.,* 2020), Peru (Sánchez *et al.* 2012; Sánchez *et al.,* 2010) and China (Ohiolei *et al.,* 2019). This may be due to the sensitivity of sheep to strain G1 *E.granulosus* and the short life span of parasite depending on sheep slaughtering age compared to other animals such as sheep, goats and cattle (Larrieu *et al.,* 2019). In addition, an essential factor that plays a key role in the spread of disease is the fertility rate in the protoscoleces in livestock farms. It was observed that the fertility rate in the protoscoleces observed in the sheep is more significant than that observed in the cystic sacs where cattle and goats live (Mokhtaria *et al.,* 2013). The phylogenetic tree that has been constructed in this study to confirm the relationship between Iraqi *E.granulosus* strain G1 and the other global isolates can be predicted. Therefore, *Echinococcus* that infect humans in Kirkuk, Iraq is a G1 sheep strain. The ND1 gene could be considered a powerful tool for accurate molecular identification and phylogenetic differentiation of this parasite.

CONCLUSIONS

E.granulosus isolates taken from sheep slaughtered in Kirkuk were identified as *E.granulosus* strain G1. These results contribute to the understanding of the epidemiology of *E.granulosus* in Iraq. The epidemiology of *E. granulosus* must be clarified in order to implement successful control programs and to help improve public health. For this reason, it is necessary to collect more information on this topic by genotyping the parasite and larval forms detected in different intermediate and definitive host populations in different geographical regions.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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